REMARKS

Claims 36, 39-43, 47, 57, 59, 60, and 89-97 are pending. Claims 89-91 were rejected under 35 U.S.C. § 112, first paragraph, and claims 36, 39-43, 47, 57, 59, 60, and 92-97 were rejected under 35 U.S.C. § 103(a).

Support for the claim amendments

Claim 36 has been amended to incorporate the features of cancelled claims 39 and 40 and now refers to isolated cells or tissue, support for which may be found, for example, on page 12, lines 24-30. In addition, claims 36, 59, and 60 are now drawn to transplantable compositions for use in humans comprising a cell or tissue having an HLA class I surface antigen. Such cells and tissues are described throughout the specification, for example, on page 12, lines 24 to 26, and page 17, lines 12 to 25. Furthermore, claim 41 has been amended to correct claim dependency. No new matter has been added by these amendments.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 89-91 were rejected under 35 U.S.C. § 112, first paragraph, on the basis that the specification fails to provide an enabling disclosure or an adequate written description of the invention, and also fails to present the best mode for carrying out the invention.

Turning to the first ground of rejection, the current Office Action asserts that the specification lacks guidance as to making and using a genetically-engineered cell. Applicant believes that this basis for the enablement rejection is unfounded.

On this point, the Examiner is directed to the specification, for example, at pages 10-12. There Applicant provides methods and published references disclosing techniques useful for genetically engineering cells having decreased class I expression. For example, under the heading of "Transgenic Animals with

Decreased HLA Class I Expression," beginning on page 10, line 3, Applicant states

> As an alternative or an adjunct to masking surface antigens on cells of donor tissues prior to transplantation, such tissues can be grown in transgenic animals which have been genetically altered so that surface antigen expression is diminished. Such transgenic animals can be made by standard transgenic techniques, employing genes which delete or inactivate the gene encoding the target antigen, or delete or inactivate a gene necessary for its expression on the cell surface, by homologous recombination. For example, in the case of HLA class I expression, homologous recombination can be used either to delete or inactivate the HLA class I molecule itself, or to inactivate or delete a companion molecule necessary for its surface expression, * * *.

Applicant further states at page 10, lines 21-25

> Inhibition of class I expression on the surfaces of cells, e.g., islet cells, can thus be achieved either by deletion or inactivation of one of the HLA class I chains, or by deletion or inactivation of the carrier β-2 microglobulin molecule, * * *.

Applicant also states, under the heading "*In Vitro* Methods to Decrease HLA Class I Expression," beginning on page 11, line 5, that

> Transfection of cultured kidney cells with fragments of adenovirus causes elimination of surface HLA class I antigenic expression, * * *.

Furthermore, under the heading "Local Blockage of Recipient T-Cell Receptors with Secreted Donor Antigens," beginning on page 11, line 28, Applicant states that

> [I]n the case of donor tissue containing parenchymal cells bearing surface HLA class I antigen, rather than masking the antigen, those cells can be transfected with DNA encoding soluble antigen, which is secreted and which competitively binds to the CD8 receptor on the T-lymphocytes of the recipient which would otherwise bind to the membrane-bound HLA class I antigen on the donor tissue cells. The techniques for carrying out this procedure will be analogous to methods used by other workers to bring about secretion of a recombinant protein in concert with insulin secretion, * * *.

These sections of the specification describe techniques that may be used to successfully genetically manipulate a cell. Disrupting a gene in a cell and transfecting DNA into a cell are clear examples of genetically engineering a cell and these techniques are standard in the art. Furthermore, support for the term "genetically-engineered" may be found on page 19, line 4, of the specification.

Turning to the assertion that Applicant's specification fails to provide an adequate written description of genetically-engineered cells, Applicant notes that the adequate written description requirement of 35 U.S.C. § 112, ¶ 1 provides:

> the specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same...

The written description requirement serves "to ensure that the inventor had possession, as of the filing date of the application relied on, of the specific subject matter later claimed by him; how the specification accomplishes this is not material." *In re Wertheim*, 541 F.2d 257, 262, 191 U.S.P.Q. 90, 96 (C.C.P.A. 1976). In order to meet the written description requirement, the applicant need not utilize any particular form of disclosure to describe the subject matter claimed, but "the description must clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed." *In re Gosteli*, 872 F.2d 1008, 1012, 10 U.S.P.Q.2d 1614, 1618 (Fed. Cir. 1989) (citation omitted). Stated another way, "the applicant must . . . convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention." *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64, 19 U.S.P.Q.2d 1111, 1117 (Fed. Cir. 1991).

As is stated above, claims 89-91 are drawn to transplantable compositions that include genetically-engineered cells where class I surface antigens have been

masked or partially or fully eliminated. Applicant's specification explicitly describes to the skilled worker what is claimed. For example, as is discussed above, Applicant explicitly describes methods for producing cells that have decreased expression of class I molecules. Clearly, Applicant was in possession of the claimed invention at the time the application was filed and provided a written description that readily enables the skilled worker to produce genetically-engineered cells falling within the claimed subject matter. There can be no question that Applicant's specification conveys clearly to those skilled in the art that the inventor has invented the claimed subject matter. Applicant's specification therefore satisfies the written description requirement of § 112. This rejection may be withdrawn.

The final basis for the present enablement rejection is the contention that, according to the Examiner, Applicant's specification fails to present the best mode contemplated by the Applicant for carrying out the invention. Regarding the requirements for rejection for lack of best mode, the MPEP (§ 2165.03, Rev. 1., February 2000) states that "the examiner should assume that the best mode is disclosed in the application, unless evidence is presented that is inconsistent with that assumption." As is noted above, Applicant's specification not only describes the claimed invention, but also teaches how to make and use the claimed genetically-engineered cells. Moreover, the Office has failed to provide evidence supporting the rejection for lack of best mode. Accordingly, this final basis for the enablement rejection should be withdrawn.

Rejections under 35 U.S.C. § 103(a)

Claims 36, 39-43, 47, 57, 59, 60, and 92-97 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Stock *et al.* (*Journal of Surgical Research* 46:317-321, 1989) in view of Faustman *et al.* (*Proc. Natl. Acad. Sci. USA* 78:5156-5159, 1981). This rejection is addressed as follows.

Stock *et al.* describe *in vitro* experiments in which murine pancreatic islet cells were treated with intact anti-class I murine monoclonal antibody and then incubated with murine T-lymphocytes. In addition, Stock *et al.* report, under "Results" on page 319, that "generation of allospecific CTL [cytotoxic T-lymphocytes] was nearly abrogated by anti-MHC class I pretreatment" and go on to suggest, in the last paragraph on page 320, that a "possibility [for preventing rejection] could involve blocking the MHC class I signal with an $F(ab)_2$ fragment of the appropriate anti-MHC class I antibody."

Applicant submits that the claimed invention is not obvious over the Stock *et al.* reference. First, Applicant's discovery that $F(ab)_2$-masked islet cells of one species could be successfully transplanted into an animal of a different, unrelated species without rejection, and with long-term maintenance of function, was totally unexpected and could not have been predicted from Stock *et al.* or any other prior art. Stock *et al.* carried out ***in vitro*** experiments, which, as is well known, frequently fail to be predictive of *in vivo* results. In the *in vivo* world, many factors could have prevented success. For example, nothing in the Stock *et al. in vitro* experiments takes into account the humoral, *i.e.,* B-cell-mediated, immune response known to play a major role in the rejection of transplanted tissue. Before Applicant carried out her experiments, it could not have been predicted that $F(ab)_2$ masking would allow successful, long-term transplantation of foreign tissue into a living animal without humoral immune system-mediated rejection. Applicant found that the $F(ab)_2$-pretreated transplanted tissue was essentially free of adjacent lymphocyte deposits 200 days following transplantation, and further found that the transplanted cells were functional after 200 days. Certainly nothing in Stock *et al.* was predictive of this dramatic result.

Furthermore, even as to the T-lymphocyte response to which Stock *et al.* confine themselves (putting aside other potential *in vivo* problems such as a likely B-cell response), Applicant's results were surprising and unexpected. In the Stock *et al.* experiment, measuring cytolytic T-cell activity was carried out immediately

following the contacting of freshly masked islet cells with lymphocytes. The abrogation of the CTL response was thus measured only in the extreme short-term; one of ordinary skill in the art would have expected that, over time, when the masking antibody dissociated from the islet cells, the CTL response would take place, particularly *in vivo*, where a limitless supply of potential CTLs is available.

Based on the results of the Stock *et al.* experiments, one of ordinary skill in the art would not have expected masking to have succeeded by substituting $F(ab)_2$ fragments for intact antibodies. Stock *et al.* used intact antibodies, which cannot be used *in vivo* because the Fc portion would fix complement and bring about lysis of the transplant. But if one were to have addressed this problem by substituting $F(ab)_2$ fragments for intact antibody molecules, one would have expected to compound the potential problem of antibody dissociating from the transplant, exposing the previously masked antigens to the host's T-lymphocytes. As was well known, $F(ab)_2$ fragments have notoriously low affinities for cell surface antigens, compared to intact antibodies. For example, Winearls *et al.* (*Transplantation* 28:36-39, 1979; copy enclosed) report that $F(ab)_2$ fragments are 100 times less effective than intact IgG in enhancement of graft tolerance, and go on to warn, in the last paragraph on page 39, that "this large difference in potency makes the clinical use of $F(ab)_2$ impractical." Thus the long-term success obtained by Applicant was all the more surprising, as it employed $F(ab)_2$ fragments.

In sum, there could have been no reasonable basis for predicting, based on the Stock *et al.* *in vitro* experiments using murine cells, that Applicant could successfully implant tissue into a human, and that this tissue would survive and remain functional for a prolonged period of time.

Turning to the Faustman *et al.* reference, Applicant notes that this reference also fails to provide a reasonable basis for predicting the success of the claimed invention. Faustman *et al.* disclose the treatment of islets with <u>anti-MHC class II</u> antibodies and complement prior to transplantation. Unlike the instant invention, which is drawn to the masking of <u>MHC class I</u> antigen on cells, the Faustman *et*

al. reference teaches the removal of MHC class II bearing cells. In Faustman *et al.*, the removal of MHC class II bearing cells was taught to be important to eliminate passenger lymphocyte cells that may be present in the graft. In contrast to MHC class II molecules which are expressed only on lymphoid cells, MHC class I molecules are present on all nucleated cells. Furthermore, the method disclosed in the Faustman et al. reference is directed toward the removal of cells by lysis using antibody and complement. As all cells in the graft express MHC class I molecules, if, instead of whole anti-class II antibodies, whole anti-class I antibodies were used in this method, the entire graft would be lysed. The instant claims are directed to the masking of class I antigens with a non-lytic masking agent that will not result in the lysis and removal of cells. Accordingly, Applicant submits that the method disclosed by Faustman et al. is irrelevant to the claimed invention.

In addition, there was no motivation to combine the teachings of Stock *et al.* and Faustman *et al.* For the reasons presented above, the *in vitro* method employed by Stock *et al.* cannot be used to accurately predict the *in vivo* effectiveness of a treatment. As a matter of fact, Applicant's specification, see for example groups 3 and 4 of Table 1, teaches that the use of whole anti-MHC class I antibodies, such as those used by Stock *et al.*, would be utterly ineffective in an *in vivo* environment. Moreover, the use of the Stock et al. method would result in accelerated rejection *in vivo*. The *in vivo* results taught by Faustman *et al.* are based on an entirely different method of pretreatment, the use of anti-MHC class II antibodies, not anti-MHC class I antibodies, than that presently claimed or that used by Stock *et al.* Consequently, one of ordinary skill in the art would not have been motivated to combine the references in the manner suggested by the Examiner and, thus, the *in vivo* success of Faustman *et al.* could not have been used to predict whether the method of Stock *et al.* would succeed *in vivo*. In view of these arguments, Applicant submits that the § 103 rejection should be withdrawn.

Information Disclosure Statement

Applicant also draws the Examiner's attention to the Information Disclosure Statement mailed on April 19, 2001 and requests that the Form PTO-1449 submitted with that statement be initialed and returned with the next Action.

CONCLUSION

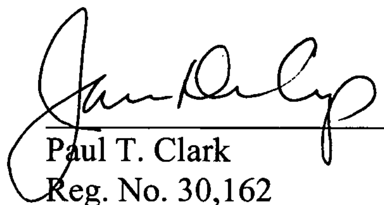
Applicant submits that the claims are in condition for allowance and such action is respectfully requested.

A marked-up version indicating the amendments made to claims 36, 41, 59, and 60, and a clean version of all pending claims reflecting entry of the amendments, are enclosed.

Also enclosed is a petition to extend the period for replying for three months, to and including October 23, 2001.

If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,



Date: 25 October 2001

Paul T. Clark
Reg. No. 30,162
James De Camp
Reg. No. 43,580

Clark & Elbing LLP
176 Federal Street
Boston, MA 02110
Telephone: 617-428-0200
Facsimile: 617-428-7045

21559
PATENT TRADEMARK OFFICE

Version with Markings to Show Changes Made

Cancel claims 39, 40, and 47.

Amend claims 36, 41, 59, and 60.

36. (Three times amended) A transplantable composition for use in humans comprising [a cell] isolated cells or isolated tissue of a type normally bearing [a] an HLA class I surface antigen that causes an immune response against the cell or tissue in a human recipient, wherein the antigen is modified, masked, or has been partially or wholly eliminated to decrease said immune response, such that upon introduction of the composition into a human, lysis of said cell or tissue is prevented; wherein

(a) said class I antigen is masked by contacting said cell or tissue with a masking agent which is capable of forming a complex with said class I antigen on said cell or tissue;

(b) said class I antigen on said cell or tissue is modified by capping; or

(c) said class I antigen on said cell or tissue is partially or wholly eliminated by inhibiting expression of said antigen on said cell or tissue.

41. (Twice amended) The composition of claim [40] 36, wherein the antigen is masked with at least two masking agents.

59. (Three times amended) A transplantable composition for use in humans comprising a cell or tissue of a type normally bearing [a] an HLA class I surface antigen that causes an immune response against the cell or tissue in a human recipient, wherein the antigen is masked such that upon introduction of the composition into a human, lysis of said cell or tissue is prevented.

60. (Three times amended) A transplantable composition for use in humans comprising a cell or tissue and at least one masking agent, wherein the masking

agent binds to [a] an HLA class I surface antigen of the cell or cells comprising the tissue that causes an immune response against the cell or tissue in a human recipient such that upon introduction of the composition into a human, lysis of said cell or tissue is prevented.

Clean Version of all Pending Claims

36. (Three times amended) A transplantable composition for use in humans comprising isolated cells or isolated tissue of a type normally bearing an HLA class I surface antigen that causes an immune response against the cell or tissue in a human recipient, wherein the antigen is modified, masked, or has been partially or wholly eliminated to decrease said immune response, such that upon introduction of the composition into a human, lysis of said cell or tissue is prevented; wherein

(a) said class I antigen is masked by contacting said cell or tissue with a masking agent which is capable of forming a complex with said class I antigen on said cell or tissue;

(b) said class I antigen on said cell or tissue is modified by capping; or

(c) said class I antigen on said cell or tissue is partially or wholly eliminated by inhibiting expression of said antigen on said cell or tissue.

41. (Twice amended) The composition of claim 36, wherein the antigen is masked with at least two masking agents.

42. (Amended) The composition of claim 41, wherein the at least two masking agents are obtained from polyclonal antisera raised against the antigen.

43. (Amended) The composition of claim 36, wherein the cell or tissue has at least two different antigens which are masked with at least two different masking agents.

57. (Amended) The composition of claim 36, which comprises a neuronal cell.

59. (Three times amended) A transplantable composition for use in humans comprising a cell or tissue of a type normally bearing an HLA class I surface antigen that causes an immune response against the cell or tissue in a human recipient, wherein the antigen is masked such that upon introduction of the composition into a human, lysis of said cell or tissue is prevented.

60. (Three times amended) A transplantable composition for use in humans comprising a cell or tissue and at least one masking agent, wherein the masking agent binds to an HLA class I surface antigen of the cell or cells comprising the tissue that causes an immune response against the cell or tissue in a human recipient such that upon introduction of the composition into a human, lysis of said cell or tissue is prevented.

89. The composition of claim 36, wherein said composition comprises a genetically engineered cell.

90. The composition of claim 59, wherein said composition comprises a genetically engineered cell.

91. The composition of claim 60, wherein said composition comprises a genetically engineered cell.

92. The composition of claim 36, wherein the cell is a non-lymphocytic cell.

93. The composition of claim 36, wherein the tissue comprises non-lymphocytic cells.

94. The composition of claim 59, wherein the cell is a non-lymphocytic cell.

95. The composition of claim 59, wherein the tissue comprises non-lymphocytic cells.

96. The composition of claim 60, wherein the cell is a non-lymphocytic cell.

97. The composition of claim 60, wherein the tissue comprises non-lymphocytic cells.

0041-1337/79/2801-0036$02.00/0
TRANSPLANTATION
Copyright © 1979 by The Williams & Wilkins Co.

Vol. 28,
Printed in U

A QUANTITATIVE COMPARISON OF WHOLE ANTIBODY AND $F(ab')_2$ IN KIDNEY ALLOGRAFT ENHANCEMENT[1]

CHRISTOPHER G. WINEARLS, JOHN W. FABRE,[2] PETER R. MILLARD, AND PETER J. MO

Nuffield Department of Surgery and Department of Pathology, University of Oxford, Radcliffe Infir
Oxford OX2 6HE, Eng

SUMMARY

Whole antiserum, IgG, and a >99% pure $F(ab')_2$ preparation were compared for their ability to enhance Lewis renal allografts in DA recipients. Despite having unimpaired antigen-binding capacity, the DA anti-Lewis $F(ab')_2$ was virtually ineffective at the highest dose tested, and was calculated to be a minimum of 100 times less effective than whole antibody. The administration of a 10-fold excess of $F(ab')_2$ before an effective dose of IgG did not block the enhancing effect of the latter.

Passive enhancement of rat renal allografts has been achieved with little evidence of damage to the graft by donor-specific antiserum. Although the rat kidney is relatively insusceptible to antibody-mediated damage produced in this way, it does occur in the DA(Ag-B[4]) to Lewis (Ag-B[1]) combination with a large dose of enhancing antiserum (1), and also in the August (Ag-B[5]) to AS (Ag-B[1]) combination in the presence of heterologous complement (2). This relative insensitivity of kidney allografts to antibody-mediated damage is not seen in other species. As the damage to the graft is initiated by complement fixation, the use of $F(ab')_2$ for enhancement has been studied by several groups (3–11).

The prevailing view is that $F(ab')_2$ can induce enhancement but that it is less effective than whole IgG. However, in most of the above studies the difference in enhancing capacity of whole IgG and $F(ab')_2$ was not adequately compared. Moreover, in many cases neither the purity of the $F(ab')_2$ nor the antigen-binding capacity after pepsin degradation were adequately established. The purity of the $F(ab')_2$ preparation is of vital importance, since it is known that minute quantities of IgG can cause enhancement (12) and, as is shown in this paper, the $F(ab')_2$ seems not to interfere with the enhancing capacity of IgG. In this study, we report the results of a quantitative comparison of the enhancing capacity for kidney allografts of a DA anti-Lewis enhancing serum and a $F(ab')_2$ preparation of known purity and binding capacity.

MATERIALS AND METHODS

Rats. Inbred male DA(Ag-B[4]) and Lewis (Ag-B[1]) rats between 10 and 20 weeks of age were used in the experiment. The animals were bred and maintained in the Nuffield Department of Surgery Animal House. Renal transplantation was performed in the Lewis to DA renal allograft model in which passive enhancement provides complete suppression of rejection (1).

Renal transplantation. The technique has been published in detail (13) but certain modifications have been made. The ureters were anastomosed end to end using four 10/0 nylon sutures. Right nephrectomy was delayed until day 7, at wh time the rat was reopened, the transplanted kidney biops and the right kidney removed. The function of the transpl was assessed by weekly blood urea estimations. The in estimation was at day 10.

Histology. Specimens were fixed in 10% formalin. Sect were stained with haematoxylin and eosin and examined w out knowledge of their origin by P. R. M.

Enhancing serum. Twenty male DA rats were immun with Lewis spleen cells. The initial injection consisted of spleen in Freund's complete adjuvant to both hind footp Boosters of 1/5 spleen in saline were injected in the f footpads at weeks 3, 7, and 12, and i.p. at week 11. Rats bled of 3 to 4 ml by cardiac puncture at weeks 8, 9, 10, 13, 14 and exsanguinated at week 15. Sera from all of these bl were pooled and the serum was coded OAS-58.

Preparation of IgG and $F(ab')_2$. IgG was precipitated f the serum three times with 18% w/v Na_2SO_4 at room temp ature. $F(ab')_2$ was prepared by pepsin digestion in 0.1 M sodi acetate buffer, pH 4.2, for 20 hr at 37 C using an enzy substrate ratio of 4% (14). After gel filtration on G-200, $F(ab')_2$ preparation showed minor contamination with u gested IgG on sodium dodecyl sulfate polyacrylamide gel el trophoresis. This contaminant was reduced by passing $F(ab')_2$ preparation through a Protein A-Sepharose 4B colu (Pharmacia, Sweden). The IgG contamination was estima to be <1% by comparison with known amounts of IgG sodium dodecyl sulfate gels. To control for experimental nipulation during the $F(ab')_2$ preparation, a portion of Na_2SO_4-precipitated IgG was incubated at pH 4.2 for 20 h 37 C but with the omission of pepsin, and also filtered on 200. The IgG was adjusted to a protein concentration of 7.6 m ml and the $F(ab')_2$ to 6 mg/ml, the protein concentrations be calculated assuming an E^{280}_{1cm} of 14.0 for 1% solutions for b the preparations (15).

Binding assay. The antigen-binding capacities of whole rum, IgG, and $F(ab')_2$ were compared using an indirect radio munoassay (16). Twenty-five microliters of varying dilution normal serum, hyperimmune serum, IgG, or $F(ab')_2$ were in bated with 25 μl of Lewis lymph node cells at 10^8/ml. This incubated for 1 hr on ice, and the cells were washed twice. Af the second wash, 100 μl of immunoadsorbent-purified rab $F(ab')_2$ anti-rat $F(ab')_2$ at 25 μg/ml and trace-labelled with were added to the cell buttons. This was incubated on ice fo hr, washed twice, and cell bound radioactivity measured i gamma counter.

Lymphocytotoxicity assay. Complement-dependent lymp cytotoxicity was measured using a ^{51}Cr release assay. T microliters of Lewis peripheral blood lymphocytes (2×10^6/ labelled with ^{51}Cr were added to 2 μl of serum, IgG, or F(ab

[1] This work was supported by a grant from the Medical Research Council and the National Kidney Foundation.

[2] Wellcome Senior Clinical Research Fellow.

dilutions in Terasaki plates and incubated for 30 min at 37 C. Five microliters of freshly thawed guinea pig complement were added and after an additional 60-min incubation, the plates were centrifuged at 250 *g* for 10 min at 4 C, and 5 μl of supernatant harvested with a Lang Levey pipette. Samples were counted in a gamma counter.

Animals were bled at day 7 after transplantation for serum lymphocytotoxic antibody measurement. Specific cytotoxicity was calculated as follows:

$$\text{Specific cytotoxicity} = \frac{E - C}{M - C} \times \frac{100}{1}$$

where E is experimental release, M is maximum release using undiluted hyperimmune serum, and C is background release with normal rat serum and complement.

The ability of $F(ab')_2$ to block the complement-dependent cytotoxicity of whole alloantiserum was tested by (1) diluting the serum in undiluted $F(ab')_2$ and (2) preincubating the target cells with undiluted $F(ab')_2$. In the latter experiment, 4×10^5 target cells were incubated in 300 μl of undiluted $F(ab')_2$ for 60 min at 4 C, washed twice, and then used as targets with whole alloantiserum and a rabbit anti-rat lymphocyte serum. Control cells were preincubated with 300 μl of normal saline. The results of this latter blocking assay are expressed as specific cytotoxicity to standardise results for the two cell preparations.

Treatment groups. The treatment groups were as follows: 1: controls received no treatment; 2: 25 μl of enhancing serum on days 0 and 1; 3: 50 μl of enhancing serum on days 0 and 1; 4: 50 μl of IgG (0.38 mg) on days 0 and 1; 5: 5 μl of IgG (0.038 mg) on days 0 and 1; 6: 500 μl of $F(ab')_2$ (3 mg) on days 0 and 1; and 7: 500 μl of $F(ab')_2$ followed ½ hr later by 50 μl of IgG on days 0 and 1.

RESULTS

Characteristics of the $F(ab')_2$ preparation. The $F(ab')_2$ preparation had completely lost its ability to induce complement-dependent cytotoxicity (Fig. 1) but its antigen-binding capacity was unimpaired (Fig. 2). The hyperimmune serum, IgG, and $F(ab')_2$ had virtually identical antigen-binding capacities. The higher binding of the hyperimmune serum at undiluted and 1/10 was probably attributable to higher backgrounds at these dilutions.

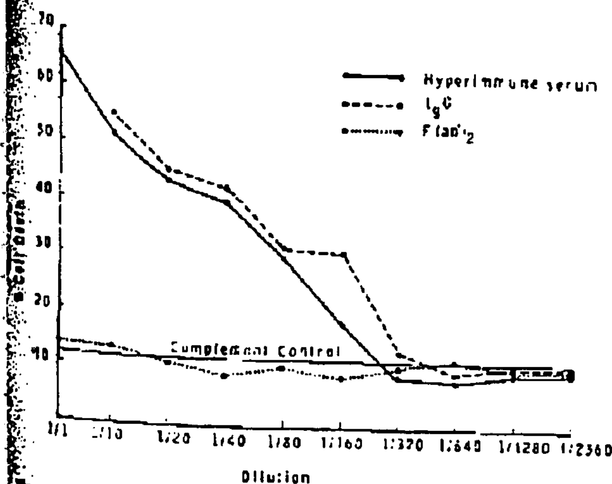


FIGURE 1. Complement-dependent lymphocytotoxic antibody titra-

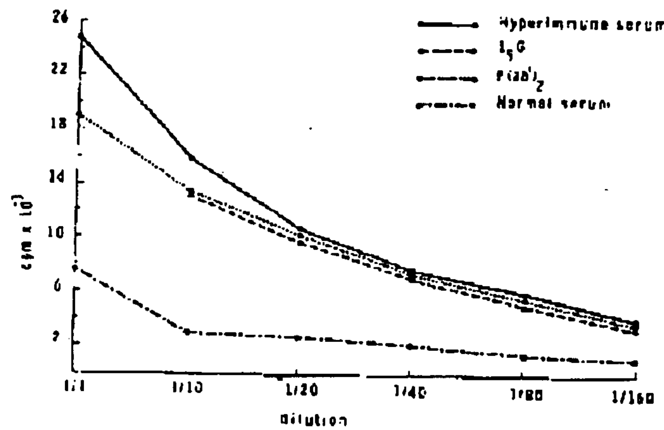


FIGURE 2. Binding assay versus Lewis lymph node cells.

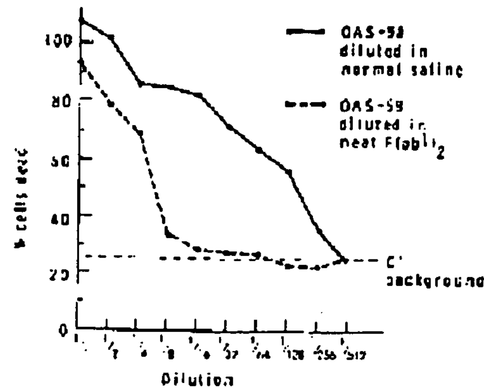


FIGURE 3. Reduction in complement-dependent cytotoxicity by $F(ab')_2$. ^{51}Cr Release assay versus Lewis peripheral blood lymphocytes.

Both dilution of the alloantiserum in $F(ab')_2$ (Fig. 3) and preincubation of the target cells with $F(ab')_2$ (Fig. 4) resulted in a significant reduction in the lymphocytotoxic antibody titre. Preincubation of the cells made no difference to the titre of the rabbit anti-rat lymphocyte serum. The inhibition of cytotoxicity by $F(ab')_2$ illustrates that negative cytotoxicity is an inadequate criterion for judging the purity of a $F(ab')_2$ preparation, since the cytotoxic titre was negative even with 1 part antiserum to 7 parts $F(ab')_2$, a contamination of >10% IgG relative to $F(ab')_2$.

Transplant results (Table 1). It can be seen that both the 50-μl and 25-μl doses of hyperimmune serum gave substantial enhancement. The less consistent results with the 25-μl dose suggest that this is approaching the minimum effective dose for this batch of serum. The 50-μl (0.38 mg) dose of IgG gave significant enhancement, although four of the five animals had rejection crises. The 5-μl dose of IgG, although less effective than the 50-μl dose, still had some immunosuppressive effect. However, the 500-μl (3 mg) dose of $F(ab')_2$ had only a trivial effect. All animals had severe acute rejection (median blood urea ± SD at 10 days = 106 ± 14 mmol/liter), from which four of six died. This dose of $F(ab')_2$ had 10 times the antigen-binding capacity of the 50-μl dose of IgG and 100 times the antigen-binding capacity of 5-μl dose. Thus, $F(ab')_2$ was approximately 100 times less effective than whole IgG.

In a further experiment we attempted to block the enhancing effect of IgG by prior administration of $F(ab')_2$. Five hundred

microliters (3 mg) of F(ab')$_2$ were administered after the release of the vascular clamps. Thirty minutes later 50 µl (0.38 mg) of IgG were administered. These doses were repeated in the same time sequence on day 1 after transplantation, and in all cases were given i.v. As can be seen from Table 1, F(ab')$_2$ did not block the enhancing effect of IgG, even though the dose of F(ab')$_2$ was 10 times that of the IgG in terms of antigen-binding capacity.

Lymphocytotoxic antibody responses (Table 2). Further evidence for the lack of effectiveness of F(ab')$_2$ can be seen from the lymphocytotoxic antibody responses to the graft. Whereas treatment with both IgG and hyperimmune serum resulted in a partial suppression of the lymphocytotoxic antibody response,

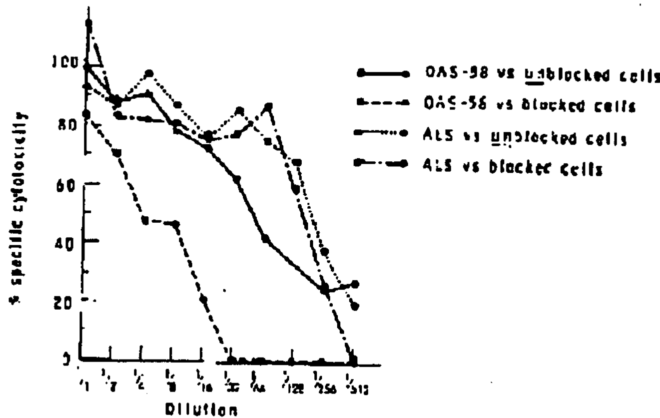


FIGURE 4. Reduction in complement-dependent cytotoxicity by preincubation of target cells with F(ab')$_2$. ^{51}Cr Release assay versus Lewis peripheral blood lymphocytes.

treatment with F(ab')$_2$ did not. The results in the ani treated with F(ab')$_2$ were similar to those of controls.

Histological results. Seven-day biopsies from untreated mals showed evidence of advanced severe rejection. The pr inent features were interstitial haemorrhage, tubular necro and glomerular capillary necrosis. Animals treated with hancing serum (25- and 50-µl doses) and IgG (50 and 5 showed a different pattern. Glomeruli and tubules were nor but, in contrast to the control kidneys, there was a mar mononuclear cell infiltrate and interstitial oedema.

Animals treated with F(ab')$_2$ showed severe acute rejecti although somewhat less severe than that of controls. There w interstitial haemorrhage, mononuclear cell infiltration, and bular necrosis but no glomerular capillary necrosis. Autop specimens from the four animals treated with F(ab')$_2$ that di showed a similar degree of rejection to that of controls, inclu ing arterial fibrinoid necrosis.

DISCUSSION

Thus, our results show that F(ab')$_2$ is approximately 100-fo less effective than whole antibody for the induction of enhanc ment of renal allografts. The F(ab')$_2$ preparation did have som slight immunosuppressive effect in that two of six rats recover from a rejection reaction and survived indefinitely. However was not possible to distinguish whether this was because of slight enhancing effect of F(ab')$_2$ or whether it was because the tiny amounts of contaminating IgG. Assuming a level of contamination of the F(ab')$_2$, the animals would have receive the equivalent of 5 µl of IgG in each 500-µl dose of F(ab')$_2$. I fact, a 5-µl dose of IgG was shown to be significantly immun suppressive. The four animals so treated survived despite acu rejection.

TABLE 1. Renal transplant results[a]

Treatment given on days 0 and 1	No. of rats	Median blood urea (mmol/liter ± SD)				Survival times (days)
		10 days	14 days	21 days	28 days	
Nil	5	127 ± 10	—	—	—	10, 10, 11, 11, 11
25 µl of enhancing serum	5	25 ± 27	16 ± 7	14 ± 6	11 ± 3	17,[b] 70,[c] >100, >100, >100
50 µl of enhancing serum	5	17 ± 2	19 ± 5	11 ± 3	11 ± 3	52,[c] >100, >100, >100, >100
5 µl of IgG (0.038 mg)	4	54 ± 21	37 ± 14	21 ± 5	18 ± 5	29[c], >42, >42, >42
50 µl of IgG (0.38 mg)	5	20 ± 13	32 ± 27	20 ± 16	18 ± 11	37, >100, >100, >100, >100
500 µl of F(ab')$_2$ (3 mg)	6	106 ± 14	30[d]	17[d]	12[d]	8, 8, 10, 11, >100, >100
Blocking experiment 500 µl of F(ab')$_2$ plus 50 µl of IgG	4	16 ± 5	18 ± 1	15 ± 2	11 ± 2	>100, >100, >100, >100

[a] Lewis → DA with 7 day nephrectomy.
[b] Unknown cause.
[c] Pneumonia.
[d] Two values only.

TABLE 2. Lymphocytotoxic antibody titrations at day 7 postgraft[a]

Treatment given i.v. on days 0 and 1	No. of rats	Mean specific cytotoxicity ± SD at dilution					
		1/1	1/2	1/4	1/8	1/16	1/32
Nil	5	89 ± 10	30 ± 13	12 ± 3	8 ± 1	4 ± 3	1 ± 1
50 µl of enhancing serum	5	50 ± 17	4 ± 6	0	0	0	0
25 µl of enhancing serum	5	42 ± 24	5 ± 7	0	0	0	0
50 µl of IgG	5	39 ± 23	8 ± 8	4 ± 2	0	0	0
5 µl of IgG	4	43 ± 19	16 ± 3	7 ± 3	4 ± 2	2 ± 1	0
500 µl of F(ab')$_2$	6	93 ± 25	31 ± 9	14 ± 6	5 ± 2	6 ± 4	1 ± 1
500 µl of F(ab')$_2$ plus 50 µl of IgG	4	60 ± 17	16 ± 7	9 ± 5	6 ± 4	5 ± 5	0

[a] DA recipients of Lewis renal allografts.

Our results show that the Fc part of the antibody molecule necessary for the induction of enhancement. It is unlikely that the digestion of the IgG resulted in any selective loss of anti-Ia-like activity because exhaustive red blood cell absorption could remove no more than 20% of the binding capacity of the $F(ab')_2$ to Lewis lymph node lymphocytes (*17*). One possibility which we did not explore is that the diminished effectiveness of $F(ab')_2$ was attributable to the much shorter half-life of $F(ab')_2$ compared to whole IgG (*18*). However, Sinclair et al. (*19*) have shown that the diminished effectiveness of $F(ab')_2$ in suppressing the antisheep erythrocyte haemolysin response cannot be attributed to the more rapid metabolism of $F(ab')_2$. Hoffmann and Kappler (*20*) have recently suggested that suppression by antibody can occur in two ways. The first is Fc independent and depends on covering of antigenic sites by antibody (or $F(ab')_2$) preventing antigenic recognition, and large doses are needed for an effect. The second is Fc dependent and prevents T and B cell cooperation by the nonspecific attachment of antigen antibody complexes to the Fc receptors of macrophages and B cells, thus taking antigen, which could stimulate helper cell activity, out of circulation.

The role of the Fc part of the IgG molecule in enhancement is unknown. Recently, Hutchinson and Zola (*21*) have postulated a mechanism which involves opsonisation of antigen-reactive cells because of antigen-antibody complexes bound to their receptor. In this model, the Fc part of the antibody would attach the whole complex to Fc receptors on the macrophage.

This large difference in potency makes the clinical use of $F(ab')_2$ impractical. However, the failure of $F(ab')_2$ to inhibit the enhancing capacity of IgG suggests that it might be possible to prevent antibody-mediated damage to a graft with $F(ab')_2$ which might limit the concentration of cytotoxic antibody on graft target cells, thereby inhibiting efficient activation of complement (*5*).

Acknowledgments. The excellent technical assistance of Joanne Gale and Elena Drexler is gratefully acknowledged.

LITERATURE CITED

1. Fabre JW, Morris PJ: 1974 Transplantation 18: 429
2. French ME: 1972 Transplantation 13: 447
3. French ME, Batchelor JR: 1972 Transplant Proc 13: 115
4. Holter AR, McKearn TJ, Neu MR, et al: 1972 Transplantation 13: 244
5. Holter AR, Neu MR, McKearn TJ, et al: 1973 Transplant Proc 5: 593
6. Myburgh JA, Smit JA: 1972 Transplantation 14: 227
7. Schilling W, Svehag SE: 1976 Acta Pathol Microbiol Scand 84: 325
8. Shaipanich T, Vanwijk RR, Kim JP, et al: 1971 Surgery 70: 113
9. Shaipanich T, Wren S, von Haefen U, et al: 1973 Transplant Proc 5: 581
10. Sutherland DER, Howard RJ, Najarian JS: 1973 Fed Proc 32: 971
11. Wren SFG, Martins ACP, von Haefen U: 1974 Surgery 76: 112
12. Fabre JW, Morris PJ: 1973 Transplantation 15: 397
13. Fabre JW, Lim SH, Morris PJ: 1971 Aust NZ J Surg 46: 69
14. Williams AF: 1975 Eur J Immunol 5: 883
15. Little JR, Donahue H: 1968, p 343 *In* Williams CA, Chase MM (eds). *Methods in immunology and immunochemistry*. Vol 2. Academic Press, Inc, NY
16. Morris RJ, Williams AF: 1975 Eur J Immunol 5: 274
17. Hart DN, Fabre JW: 1979 Transplantation 27: 110
18. Spiegelberg HL, Weigle WO: 1965 J Exp Med 121: 323
19. Sinclair JRStC, Lees RK, Chan PL, et al: 1970 Immunology 19: 105
20. Hoffman MK, Kappler JW: 1978 Nature 272: 64
21. Hutchinson IV, Zola H: 1977 Transplantation 23: 464

Received 24 January 1979.
Accepted 29 January 1979.